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(54) Title: PRODUCTION OF RECOMBINANT HUMAN LACTOFERRIN

(57) Abstract

The present invention provides novel plasmids, transfected eucaryotic cells and methods of producing these plasmids and transfected eucaryotic cells. The novel plasmid contains the cDNA for human lactoferrin protein. Methods for the production of human lactoferrin protein in *A. Oryzae* are also provided. Thus, the present invention provides an efficient and economical means for the production of recombinant human lactoferrin protein.

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PRODUCTION OF RECOMBINANT HUMAN LACTOFERRIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the field of iron-binding glycoproteins. More specifically, the present invention relates to the recombinant production of human lactoferrin.

Description of the Related Art

Human lactoferrin (LF) is a member of the transferrin family of iron-binding monomeric glycoproteins. It was originally discovered in milk where it can reach levels of 7 grams/liter in colostrum. LF has since been detected in other external fluids such as tears, saliva and mucosal secretions and also in the secondary granules of polymorphonuclear leukocytes.

LF is a 78 kDa glycoprotein having a bilobal structure with a high degree of homology between the C and N terminal halves which is evident at both the amino acid and three dimensional structural level. Each of these lobes can reversibly bind one ferric iron with high affinity and with the concomitant binding of bicarbonate. The biological functions proposed for lactoferrin include protection against microbial infection,

enhanced intestinal iron absorption in infants, promotion of cell growth, regulation of myelopoiesis and modulation of inflammatory responses.

Filamentous fungi have been successfully employed as hosts in the industrial production of extracellular glycoproteins. Certain industrial strains are capable of secreting gram quantities of these proteins. In addition, filamentous fungi are able to correctly perform post-translational modifications of eucaryotic proteins and many strains have U.S Food and Drug Administration approval. Furthermore, large scale fermentation technology and downstream processing experience is available.

Currently, there is no efficient and economical way to produce human LF. Consequently, a long felt need and description in this art would be met by the development of an efficient method for the production of human lactoferrin for nutritional and therapeutic applications and for further investigation into its mechanism of action.

SUMMARY OF THE INVENTION

In one embodiment, the present invention provides for a recombinant plasmid comprising the cDNA of human lactoferrin. The plasmid of the present invention is adapted for expression in an eucaryotic cell and contains the regulatory elements necessary for the expression of the human lactoferrin cDNA in this eucaryotic cell.

In another embodiment, the present invention provides for a transformed eucaryotic cell which includes a recombinant plasmid. The eucaryotic cell is selected from a group of filamentous fungi comprising Aspergillus. The plasmid contains a plasmid vector into which a polydeoxyribonucleotide segment coding for human lactoferrin protein has been inserted.

In yet another embodiment of the present invention, there is provided a process for producing recombinant human lactoferrin which comprises culturing a transformant eucaryotic cell, which includes a

recombinant plasmid. The plasmid contains a plasmid vector having a polydeoxyribonucleotide coding for the human lactoferrin protein. After culturing in a suitable nutrient medium until human lactoferrin protein is formed, the human lactoferrin protein is isolated.

In still yet another embodiment of the present invention, there is provided a recombinant expression vector. This vector comprises a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression; (2) cDNA coding for human lactoferrin; (3) appropriate transcription and translation initiation and termination sequences; and (4) a genetic element for selection of aspergillus spores that have been transformed with the vector.

In still yet another embodiment of the present invention, there is provided a method for producing biologically active recombinant lactoferrin. The method comprises synthesizing sequences containing a selectable marker gene, a promotor, a transcription termination sequence, and a linker sequence; cloning the sequences to form a plasmid; digesting the plasmid with a restriction endonuclease; inserting a cDNA coding for lactoferrin into a restriction site; and transforming eucaryotic cells with the plasmid expressing lactoferrin cDNA.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the manner in which the above recited features, advantages, and objects of the invention, as well as others which will become clear, are obtained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of this specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore not to be considered limiting of its scope. The invention may admit to other equally effective equivalent embodiments.

Fig. 1 depicts a schematic representation of the *aspergillus oryzae* expression plasmid, pAhlfg.

Fig. 2 shows a southern blot analysis of transformed *aspergillus oryzae* strains.

Fig. 3 depicts an RNA analysis of transformant versus control A07.

Fig. 4 shows the silver stained SDS-acrylimide gel analysis of recombinant LF secretion and purification.

Fig. 5 illustrates the characterization of recombinant human LF.

Fig. 6 depicts the cDNA sequence for human lactoferrin.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

For the purposes of the present application, the term "transferrin family" means a family of iron transferring proteins including serum transferrin, ovotransferrin and lactoferrin. These proteins are all structurally related.

For the purposes of the present application, the term "vector(s)" means plasmid vehicle to allow insertion, propagation and expression of lactoferrin cDNA.

For the purposes of the present application, the term "host(s)" means any eucaryotic cell that will allow integration of the lacatoferrin expression plasmid into its genome.

For the purposes of the present application, the term "promotor(s)" means regulatory DNA sequences that controls transcription of the lactoferrin cDNA.

For the purposes of the present application, the term "multiple cloning cassette" means a DNA fragment containing restriction enzyme cleavage sites for a variety of enzymes allowing insertion of a variety of cDNAs.

For the purposes of the present application, the term "transformation" means uptake of plasmid by a relevant eucaryotic cell.

For the purposes of the present application, the term "iron binding capacity" means ability to bind ^{59}Fe. Fully functional lactoferrin can bind two atoms of iron per molecule of LF.

For the purposes of the present application, the term "biological activity/biological active" means biological activity of lacatoferrin as measured by its ability to bind iron. The lactoferrin protein functions as an iron transfer protein and must bind iron to be biologically active.

All literature references cited in this specification are hereby expressly incorporated by reference.

The following examples are given for the purposes of illustrating various embodiments of the present invention and are not meant to be limitations of the present invention in any form.

Example 1

Fungal strains and transformation

The pyrG mutant strain used in these studies was derived from *A. oryzae* (A07 11488). The pyrG gene from *A. oryzae* was mutated with 4-nitroquinoline-1-oxide. The Aspergillus transformation was carried out by a modification of the procedure of Osmani, et al., *J. Cell. Biol.* 104:1495-1504 (1987). Conidia ($1X10^6$/ml) were inoculated into 50 ml of YG medium (0.5% yeast extract 2% glucose) containing 5 mM uracil and 10 mM uridine. Growth was at 32°C for 14-16 hours until a germ tube was visible. The germinated conidia were harvested by centrifugation and resuspended in 40 m1 of lytic mix containing 0.4 M ammonium sulphate, 50 mM potassium citrate (pH 6.0), 0.5% yeast extract, 0. 12 g novozyme, 0.1g Driselase, 100 µl β-glucuronidase, 0.5% sucrose and 10 mM $MgS0_4$. Protoplasting was for 2-3 hours at 32°C and 150 rpm. Following protoplasting, filtration using sterile miracloth was necessary to remove

any undigested mycelia. The protoplasts were harvested by centrifugation and washed twice with 10 ml of 0.4 M ammonium sulphate, 1% sucrose and 50 mM potassium citrate (pH 6.0) at 4°C, resuspended in 1 ml of 0.6 M KCl; 50 mM CaCl; 10 mM Tris-HC1 (pH 7.5) and placed on ice. The transformation was performed immediately following the protoplast preparation. Aliquots (100 μl) of the protoplast were added to 3 μg of DNA and 50 μl of 40% polyethylene glycol (PEG) 6000, 50 mM $CaCl_2$, 0.6 M KCl and 10 mM Tris-HCl,(pH 7.5). The samples were incubated on ice for fifteen minutes after which an additional 1 ml of the PEG solution was added and incubation at room temperature was continued for thirty minutes. Aliquots of this mixture were plated in 3 mls of 0.7% minimal media, supplemented with 0.4% ammonium sulphate onto plates containing the same but solidified with 2% agar. All subsequent growth was at 32°C.

Example 2
Plasmid Construction

A schematic representation of the expression plasmid is shown in Fig. 1. The complete cDNA encoding human LF was repaired using the Klenow fragment of DNA polymerase I and subcloned into AccI digested and repaired pGEM4 to generate pGEMhLFc. In order to remove the LF signal sequence and generate a 5' end in frame with the α-amylase sequences, a 252 base pair lactoferrin fragment (nt 69-321) containing HindII/AccI ends was obtained by polymerase chain reaction (PCR) amplification of pGEMhLFc plasmid DNA. The oligo primers used were as follows: the 5' end oligonucleotide as shown in SEQ. ID. No. 1:

(CTGGGTCGACGTAGGAGAAGGAGTGTTCAGTGGTGC)

and the 3' end oligonucleotide as shown in SEQ. ID. No. 2:

(GCCGTAGACTTCCGCCGCTACAGG).

This PCR fragment was digested with HindII and AccI and was subcloned into Hind 11/AccI digested pGEMhLFC generating pGEMhLF. A 681 base pair α-amylase fragment with Asp718/PvuII ends encoding the promotor, signal sequence and the alanine residue from the start of the mature α-amylase II gene, was obtained by PCR amplification of *A. oryzae* genomic DNA. The oligo primers were as follows: the 5'end oligonucleotide as shown in SEQ. ID. No. 3:

(GAGGTACCGAATTCATGGTGTTTTGATCATTTTAAATTTTTATAT)

and the 3'end oligonucleotide as shown in SEQ. ID. No. 4:

(AGCAGCTGCAGCCAAAGCAGGTGCCGCGACCTGAAGGCCGTACAG).

The amplified DNA was digested with Asp718 and PvuII and subcloned into Asp718/Hind11 digested pGEMhLF. The resulting plasmid (pGEMAhLF) was digested with EcoRI and the resulting 2.8 kb α-amylase-lactoferrin fragment was subcloned into a unique EcoRI site in pAL3 according to the method of generating pAhLF*. Synthetic oligonucleotide were used to provide the last five carboxy terminal codons of lactoferrin (nt 2138 - 2153) missing in pAhLF* and also to provide the first 180 bp of 3' untranslated sequences from the A. niger glucoamylase gene. The resulting plasmid (pAhLFG) was used to transform the *A. oryzae* pyrG mutant strain.

With reference to FIGURE 1, Aspergillus *oryzae* expression plasmid, pAhLFG contains 681 bp of 5'-flanking sequence of the *A. oryzae* AMY11 gene which includes the signal sequence and first codon of mature α-amylase. The cDNA coding for mature human lactoferrin is subcloned in frame downstream from these sequences allowing recombinant protein

production by the addition of starch to the growth medium. The Aspergillus *niger* glucoamylase 3' untranslated region provides the transcription terminator and polyadenylation signals. The plasmid also contains the Neurospora crassa pyr4 selectable marker and an ampicillin resistance gene.

The plasmid construct (pAhLFG) used for expression of human LF contains a 681 bp fragment that encodes the promotor and secretory signal peptide of the *A. oryzae* α-amylase II gene (AMY11). The signal sequence also contains the codon for alanine from the start of the α-amylase mature protein generating the signal sequence cleavage site (Leu Ala Ala) recognizable by an endogenase α-amylase peptidase. A human lactoferrin cDNA fragment encoding the mature protein was subcloned in frame immediately downstream from the AMYII sequences, placing it under the control of this highly efficient starch inducible promoter. In order to stabilize the transcribed human LF mRNA, a 180 bp fragment encoding the 3' untranslated region of the glucoamylase gene from Aspergillus niger was ligated into a unique BamHI site in the multiple cloning cassette, immediately downstream of the human LF cDNA providing the transcription terminator and polyadenylation signals. The plasmid also contains the Neurospora crassa Pyr4 selectable marker which complements a pyrG auxotrophic mutation of *A. oryzae* and allows for selection of spores that have been transformed with the plasmid by growth in the absence of uridine.

Example 3

Genomic DNA Manipulation

A. oryzae DNA was isolated from 200 mg of lyophilized mycelia as described by Rafmussen, et al., *J. Biol. Chem.*, 265:13767-13775 (1990). The DNA was digested with EcoRI, size fractionated on a 0.8% agarose gel and transferred to nitrocellulose. Prehybridization and hybridization of the nitrocellulose filter for Southern analysis were

performed in 6XSSC, 0.1% SDS and 0.5% dried milk at 65°C for 16 hours. Hybridization solution contained 1 x 10^7 cpm ^{32}P-labelled lactoferrin cDNA probe (2.1 Kb). The filter was washed in 2XSSC, 0.5% SDS at room temperature for 30 minutes followed by two washes in 0.5X SSC, 0.5% SDS at 68°C for 30 minutes. The filter was dried, exposed at -70°C for two hours and developed by autoradiography.

With reference to FIGURE 2, Southern blot analysis was performed on transformed Aspergillus *oryzae* strains. Genomic DNA from individual transformants and control AO7 were hybridized with a radiolabelled hLF cDNA probe (2.1 kb). The arrow points to a radiolabelled fragment (2.8 kb) generated upon EcoR1 digestion of the expression plasmid which is present in all the transformants (#1-9) but is absent in control untransformed AO7. Molecular weights of bacteriophage lambda Hind 111 fragments are indicated at the left.

Example 4
Northern Analysis

RNA was isolated from lyophilized mycelia (200 mg) using commercially available RNazol B (Biotecx Laboratories, INC, Houston, TX) according to the manufacturers instructions. Total RNA (20 µg) was electrophoresed in a 0.8% agarose gel containing 2.2 M formaldehyde. The RNA was transferred to nitrocellulose and hybridized with either a 2.1 kb lactoferrin cDNA or a 1.8 kb genomic α-amylase fragment corresponding to the coding region of the α-amylase 11 gene. The probes were ^{32}P-labelled by nick translation (specific activity 2 X 10^8 cpm/ug). Hybridization was carried out 2 x SSC, .05% dried milk at 65°C over an ice with 2 x 10^6 cpm probe/ml.

Washes were identical to those employed in the Southern analysis. The filters were dried, exposed at -70°C for two hours and developed by autoradiography. RNA dot blots were performed using nitrocellulose membrane and the manifold dot blot system. Hybridization

and washing conditions were as described above for Southern analysis. Radioactivity was quantitated using the betagon blot analyzer.

Recombinant production of lactoferrin protein has been described in its preferred embodiment. However, it could also be produced in a number of other sources such as fungal sources such as saccharomyces cerevisiae or pichia pastorsis or insect cells such as SF9.

With reference to FIGURE 3, RNA analysis of transformant versus control AO7 was performed. In Panel A, Northern analysis of RNA (20 μg) from control AO7 and transformant #1 hybridized with radiolabelled human LF cDNA. Human LF mRNA (2.3 kb) was detected in the transformant #1 but not in the control untransformed AO7. The positions of the 28s and 18s rRNA bands are indicated on the left. In Panel B, Dot blots of RNA (5 and 10 μg) from control AO7 versus transformant #1 using a radiolabelled α-amylase genomic DNA probe. In Panel C, Dot blots of RNA (5 and 10μg from control AO7 and transformant #1 using radiolabelled human LF cDNA probe as illustrated.

Northern analysis was performed to determine if lactoferrin mRNA was transcribed correctly and efficiently in *A. oryzae* under the regulatory control elements of our expression plasmid. Spores ($1x10^6$/ml) from transformant #1 and from control untransformed spores were inoculated into fungal medium containing 1.5% glucose as carbon source and grown at 30°C for 48 hours in small shake flask cultures. The cultures were washed and reinoculated into fungal medium containing 3% starch to induce transcription of the human LF mRNA. After 24 hours, the cells were harvested and RNA was isolated. Total RNA (20 μg) was size fractionated on a 1.0% agarose gel containing 2.2 M formaldehyde and blotted on nitrocellulose.

Human lactoferrin mRNA was detected using ^{32}P labelled human LF cDNA (2.O kb) probe. Hybridization with human LF radiolabelled cDNA probe detected a specific radiolabelled band at the correct size for lactoferrin mRNA (2.3kb) in the transformant but not in

the control untransformed strain (Fig. 3A). Quantitation of mRNA levels by dot assay showed comparable levels of expression of endogenous α-amylase rRNA between control AO7 and transformant #1 (Fig. 3B). In addition, similar levels of expression of α-amylase and human LF mRNA were seen in transformant #1 (Fig.3B and 3C).

Example 5

Purification of Recombinant human LF

LF was purified from the growth medium using CM Sephadex C50 essentially as described by Stowell, et al., *Biochem J.*, 276:349-59 (1991). The column was preequilibrated with 500 ml of 0.025 M Tris HCl, pH 7.5O 1M NaC1. The pH of the culture medium was adjusted to pH 7.4 before applying to the preequilibrated column. The column was washed with 500 ml of equilibration buffer and followed by a linear salt gradient from 0.1 to 1.1 M NaC1. Fractions (7 ml total) were assayed for lactoferrin content and purity using SDS/PAGE and silver staining. Fractions containing LF were dialyzed against 0.025 M Tris HCl, pH 7.5/0.1M NaCl and lyophilized.

Example 6

Quantitation of human LF

Recombinant lactoferrin was quantitated using an ELISA assay essentially as described by Vilja et al., *J. Immunol. Methods*, 76:73-83 (1985). A sensitivity of 5 ng of lactoferrin was obtained using the non-competitive Avidin-biotin assay. Human LF isolated from breast milk (Sigma) was used as standard. Biotinylated human lactoferrin IgG was obtained from Jackson Immunoresearch laboratories, West Grove, PA.

Example 7

N-terminal Sequencing

Five µg of purified recombinant human LF was resolved on an SDS-polyacrylamide gel and transferred to Problott, a polyvinylidene difluride-type membrane, following manufacturers instructions (Applied Biosystems). Human LF was detected with Comassie Brilliant Blue staining and destained. This human LF band was excised, washed thoroughly with distilled H_20 and air-dried. The N-terminal amino acid sequence of the first ten amino acids of human LF was determined by the automated Edman degradation procedure using an applied Biosystems Pulsed-liquid phase sequencer (Model 477A).

With reference to FIGURE 4, panel A illustrates a Silver stained SDS-polyacrylamide gel analysis of recombinant human LF secretion and purification. Lane 1 contains breast milk human LF standard (500 ng). Lanes 2 and 3 contain samples of the growth medium (40 µg) from induced control AO7 and transformant #1 respectively. Lanes 4-8 contain 100 µl aliquots of eluted fractions (#25, 30, 35, 40, and 45 respectively) collected from the CM-sephadex purification of recombinant LF from the growth medium of transformant #1. The position of the molecular weight markers (BioRad laboratories Richmond, CA) are indicated on the left. Sizes are given in kilodaltons. Panel B illustrates a Western immunoblot analysis of duplicate samples as described in panel A using a specific polyclonal antibody directed against human LF with detection with ^{125}I-protein A. Panel C illustrates #6 N-terminal amino acid sequence of recombinant human LF. Recombinant human LF was sequenced from the N-terminus through 10 residues and is identical to breast milk human LF with the exception of the additional alanine generated in our construction to provide the α-amylase signal sequence cleavage site.

Example 8

Deglycosylation

Deglycosylation was performed using N-glycosidase F (Boehringer Mannheim). *A. oryzae* growth medium containing 0.5 μg lactoferrin was denatured for 3 minutes at 100° C in the presence of 0.01% SDS. Standard LF from human milk was treated similarly. The samples were subsequently placed on ice for five minutes. N-glycosidase F reactions were conducted in 0.4 M sodium phosphate, (pH 6.8); 0.08% Triton; 0.1% β-mercaptoethanol and 1 unit of enzyme and incubated at 37° C for sixteen hours. PAGE and western analysis was performed using an IgG specifically directed against human lactoferrin to detect an increase in mobility of digested samples.

With reference to FIGURE 5, recombinant human LF was characterized. Panel A illustrates the deglycosylation of lactoferrin. Western analysis of glycosylated and deglycosylated lactoferrin using a specific polyclonal antibody was directed against human lactoferrin with detection with ^{125}I-protein A. The first panel contains authentic breast milk human LF (500 ng) untreated (-) and treated (+) with N glycosidase F. The second panel contains purified recombinant human LF (500 ng) untreated (-) and treated (+) with N-glycosidase F. The size of glycosylated human LF is indicated with the arrow. Panel B illustrates a functional analysis of recombinant lactoferrin with regard to iron-binding capacity. Panel A and B show the ^{59}Fe filter binding assay of duplicate samples of authentic breast milk human LF and purified recombinant human LF, respectively, at the concentrations indicated. The first lane in both panels contain BSA (5 μg) as a negative control.

Lactoferrin contains two N-acetyllactamine type glycans attached through N-glycosidic linkages. To determine if recombinant lactoferrin was glycosylated correctly, the protein was treated with N-glycosidase F, resolved on SDS-polyacrylamide electrophoresis, transferred to nitrocellulose and probed using a specific IgG directed against human

lactoferrin (Fig. 5A). N-glycosidase F hydrolyses at the glycosylamine linkage generating a carbohydrate free peptide of smaller molecular weight. Comparison of recombinant LF with purified LF from human milk, illustrates that both proteins co-migrate upon digestion with N-glycosidase F suggesting that the recombinant protein has a glycosylation pattern similar to native LF.

Lactoferrin has a bilobal structure with each lobe having the capacity to bind tightly, but reversibly, one Fe^{3+} ion. The iron-binding properties of lactoferrin are crucial for its functional roles. To test if recombinant human LF expressed and secreted in *A. oryzae* has an iron binding capacity similar to authentic lactoferrin, an ^{59}Fe micro filter binding assay was developed. Purified human lactoferrin isolated from the growth medium of transformant #1 was dialyzed against 0.1M citric acid (pH 2.0) to generate apo-human LF. Native lactoferrin from human milk was treated similarly. Excess ^{59}Fe (0.2 mCi) was added to these samples in an equal volume of 1 M bicarbonate, followed by incubation at 37°C for 30 minutes. Samples were applied to nitrocellulose membrane and washed several times with bicarbonate. The filter was visualized by autoradiography and Fe-binding was quantitated using a betagon blot analyzer. As illustrated in Fig. 5B, both recombinant and native LF showed a similar level of iron binding at all concentrations tested. The results demonstrate that recombinant human LF Is indistinguishable from native human LF in its capacity to bind iron.

With reference to Figure 6, the complete cDNA sequence for human lactoferrin protein is depicted. The cDNA coding for lactoferrin is used to create plasmids and transform eucaryotic cells and to produce the lactoferrin protein.

Strains of aspergillus used in the present invention are auxotrophic mutants that contain a defective pry 4 gene that results in an inability to synthesis orotidine 5' phosphate (OMP) decarboxylase. The enzyme is required for uridine synthesis. The strain cannot grow on

media lacking uridine. The plasmid contains a selectable marker, i.e., a sequence that encodes the gene for OMP decarboxylase. Uptake of the plasmid by the aspergillus can therefore be selected for by growth on media lacking uridine. The aspergillus is transformed by the plasmid such that it can grow on the uridine deficient media.

In one embodiment of the present invention, biologically active recombinant lactoferrin protein is produced. This method comprises synthesizing sequences containing a selectable marker gene, a promotor, a transcription termination sequence and a linker sequence. Subsequently, the sequences are cloned to form a plasmid and the plasmid is digested with a restriction endonuclease. A cDNA coding for lactoferrin is inserted into a restriction site and eucaryotic cells are then transformed with the plasmid expressing the lactoferrin cDNA.

The selectable marker gene useful in the method of the present invention may be any that permits isolation of cells transformed with a lactoferrin cDNA plasmid. Preferably, the selectable marker gene is selected from pyr4, pyrG, argB, trpC and andS.

The promotor useful in the present invention may be any that allows regulation of the transcription of the lactoferrin cDNA. Preferably, the promotor is selected from the group of alcohol dehydrogenase, argB, α-amylase and glucoamylase.

The transcription termination sequence useful in the present method may be any that allows stabilization of the lactoferrin mRNA. Preferably, the transcription termination sequence is derived from α-amylase, glucoamylase, alcohol dehydrogenase or benA.

The linker sequence useful in the present method may be any that contains a translation initiation codon, a secretory signal and a restriction enzyme cleavage site. Preferably, the linker element is derived from α-amylase, glucoamylase or lactoferrin.

The eucaryotic cells useful in the present invention are any that allow for integration of a plasmid comprising the lactoferrin cDNA

and expression of the lactoferrin cDNA. Preferably, the eucaryotic cells are fungal cells or insect cells. Insect cells such as SF9 are useful in the method of the present invention. More preferably, the fungal cells are yeast cells. Most preferably, the eucaryotic cells useful in the present invention are aspergillus strains, such as A. *oryzae*, A. *Niger*, A. *Nidulans* and A. *Awamori*.

In conclusion, it is seen that the present invention and the embodiments disclosed herein are well adapted to carry out the objectives and obtain the end set forth in this application. Certain changes can be made in the method and apparatus without parting from the spirit and scope of this invention. It is realized that changes are possible and that it is further intended that each element or step presided in any of the filing claims is to be understood as to referring to all equivalent elements or steps for accomplishing the essentially the same results in substantially the same or equivalent manner. It is intended to cover the invention broadly in whatever form its principles may be utilized. The present invention, therefore, is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as others inherent therein.

What is claimed is:

1. A method for producing biologically active recombinant lactoferrin comprising the steps of:

combining sequences containing a selectable marker gene, a promotor, a transcription termination sequence, and a linker sequence;

cloning said sequences to form a plasmid;

digesting said plasmid with a restriction endonuclease;

inserting a cDNA coding for lactoferrin into a restriction site; and

transforming eucaryotic cells with said plasmid expressing lactoferrin cDNA.

2. The method of Claim 1, wherein said selectable marker gene is selected from the group consisting of pyr4, pyrG, andS, argB and trpC.

3. The method of Claim 1, wherein said cell expresses lactoferrin.

4. Lactoferrin produced by the method of Claim 2.

5. The method of Claim 1, wherein said promotor is selected from the group consisting of alcohol dehydrogenase, argB, α-amylase, glucoamylase, and benA.

6. The method of Claim 1, wherein said transcription termination sequence is selected from the group consisting of α-amylase, glucoamylase, alcohol dehydrogenase and benA.

7. The method of Claim 1, wherein said linker sequence is selected from the group consisting of α-amylase, glucoamylase and lactoferrin.

8. A plasmid adopted for expression in a eucaryotic cell which comprises the cDNA of Figure 6 and the regulatory elements necessary for the expression of the cDNA in the eucaryotic cell.

9. A plasmid designated pAhLFG.

10. A eucaryotic cell containing the plasmid of Claim 8.

11. The eucaryotic cell of Claim 10, wherein said eucaryotic cell is selected from the group consisting of fungi and insect cells.

12. The eucaryotic cell of Claim 11, wherein said insect cell is SF9.

13. The eucaryotic cell of Claim 11, wherein said fungal cell is yeast.

14. The eucaryotic cell of Claim 13, wherein said yeast cell is aspergillus.

15. The eucaryotic cell of Claim 14, wherein said strain of aspergillus is selected from the group consisting of A. *oryzae*, A. *Niger*, A. *Nidulans* and A. *Awamori*.

16. A process for producing lactoferrin which comprises culturing a transformant eucaryotic cell containing a recombinant plasmid, said plasmid comprising a plasmid vector having a polydeoxyribonucleotide which codes for lactoferrin proteins in a suitable nutrient medium until lactoferrin protein is formed and, isolating the human lactoferrin.

17. A recombinant expression vector having a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role

in gene expression; (2) cDNA coding for human lactoferrin; and (3) appropriate transcription and translation initiation and termination sequences.

18. The vector of Claim 17, wherein said genetic element is a promotor.

19. The vector of Claim 18, wherein said promotor is selected from the group consisting of alcohol dehydrogenase, argB, α-amylase, glucoamylase, and benA.

20. The vector of Claim 17, wherein said transcription termination sequence is selected from the group consisting of α-amylase, gluocoamylase, alcohol dehydrogenase and benA.

21. The protein product of Claim 16.

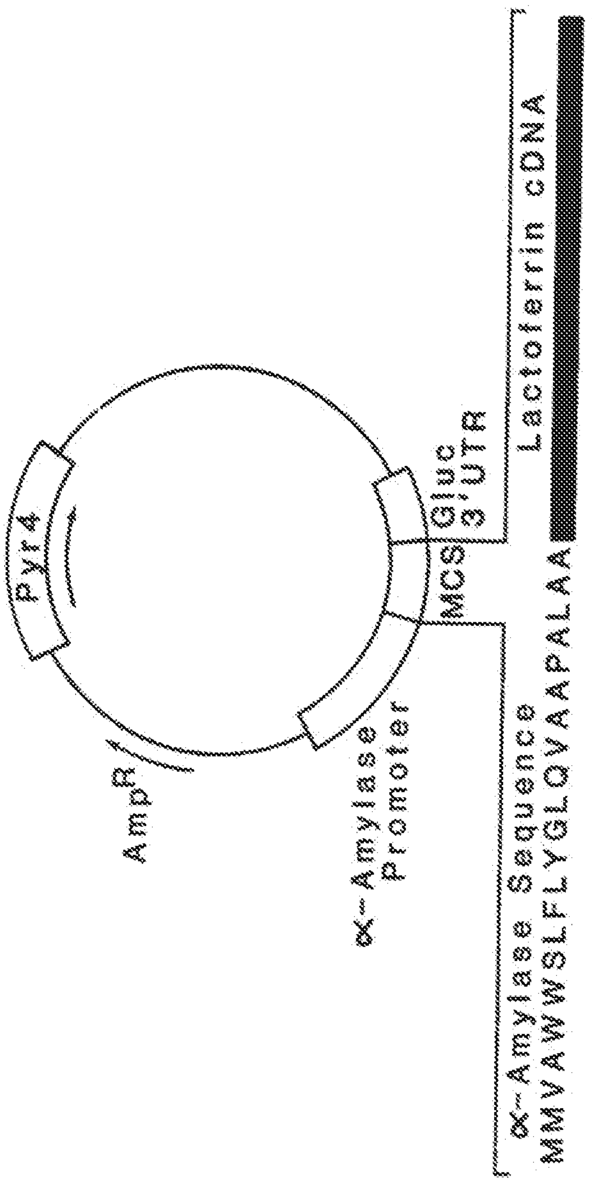

Pyr4
Amp^R
α-Amylase Promoter
MCS
Gluc 3'UTR
Lactoferrin cDNA
α-Amylase Sequence
MMVAWWSLFLYGLQVAAPALAA

FIG. 1

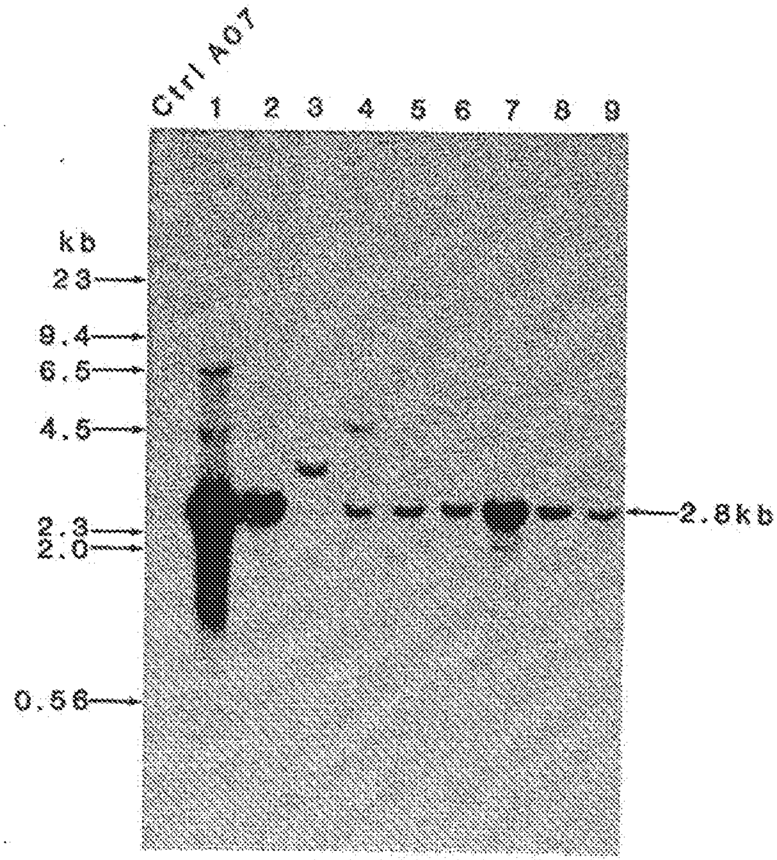


FIG. 2

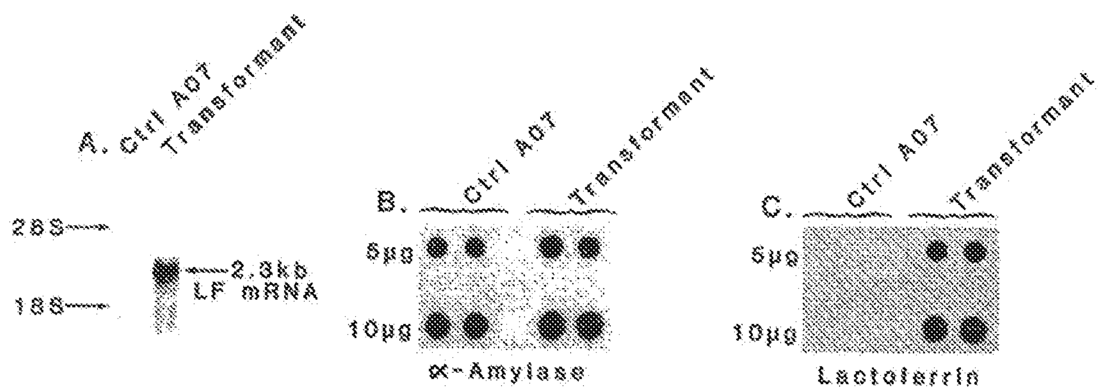


FIG. 3A FIG. 3B FIG. 3C

FIG. 4A

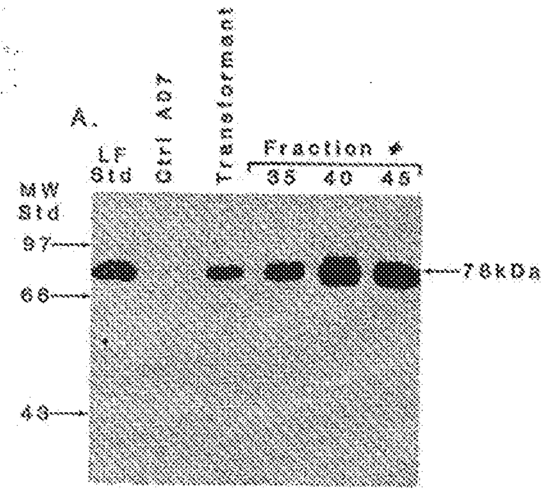
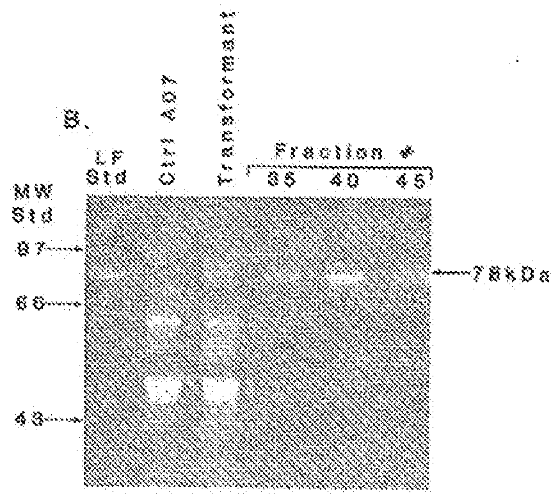


FIG. 4B



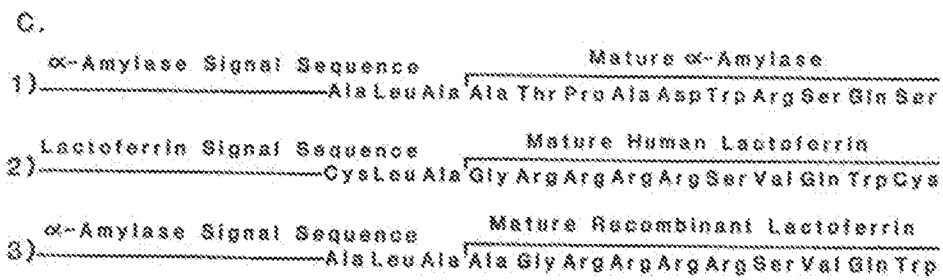


FIG. 4C

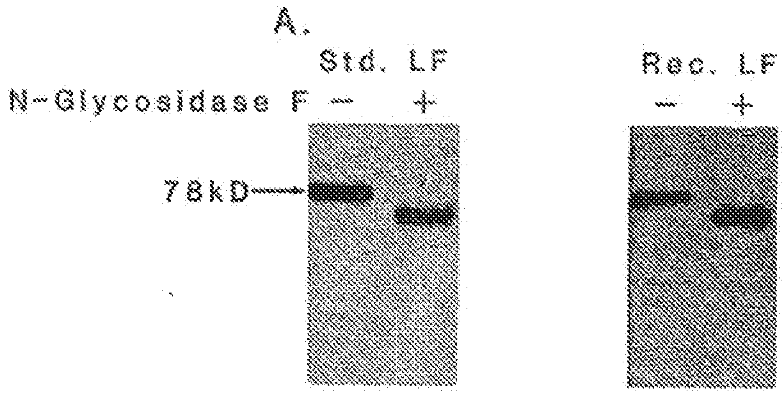

A.
Std. LF
Rec. LF
N-Glycosidase F
−
+
−
+
78kD

FIG. 5A

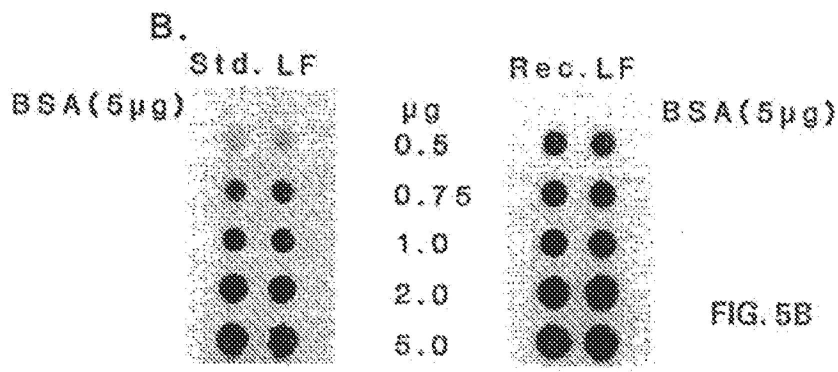

B.
Std. LF
Rec. LF
BSA(5µg)
µg
0.5
0.75
1.0
2.0
5.0
BSA(5µg)

FIG. 5B

cDNA SEQUENCE AND AMINO ACID SEQUENCE
OF HUMAN LACTOFERRIN

```
                                                       1
                                                       GAATTCC GACCGCAGAC
18
ATG AAA CTT GTC TTC CTC GTC CTG CTG TTC CTC GGG GCC CTC GGA CTG
met lys leu val phe leu val leu leu phe leu gly ala leu gly leu
 1
66
TGT CTG GCT GGC CGT AGG AGA AGG AGT GTT CAG TGG TGC ACC GTA TCC
cys leu ala gly arg arg arg arg ser val gln trp cys thr val ser
 17
114
CAA CCC GAG GCC ACA AAA TGC TTC CAA TGG CAA AGG AAT ATG AGA AGA
gln pro glu ala thr lys cys phe gln trp gln arg asn met arg arg
 33
162
GTG CGT GGC CCT CCT GTC AGC TGC ATA AAG AGA GAC TCC CCC ATC CAG
val arg gly pro pro val ser cys ile lys arg asp ser pro ile gln
 49
210
TGT ATC CAG GCC ATT GCG GAA AAC AGG GCC GAT GCT GTG ACC CTT GAT
cys ile gln ala ile ala glu asn arg ala asp ala val thr leu asp
 65
258
GGT GGT TTC ATA TAC GAG GCA GGC CTG GCC CCC TAC AAA CTG CGA CCT
gly gly phe ile tyr glu ala gly leu ala pro tyr lys leu arg pro
 81
306
GTA GCG GCG GAA GTC TAC GGG ACC GAA AGA CAG CCA CGA ACT CAC TAT
val ala ala glu val tyr gly thr glu arg gln pro arg thr his tyr
 97
354
TAT GCC GTG GCT GTG GTG AAG AAG GGC GGC AGC TTT CAG CTG AAC GAA
tyr ala val ala val val lys lys gly gly ser phe gln leu asn glu
 113
402
CTG CAA GGT CTG AAG TCC TGC CAC ACA GGC CTT CGC AGG ACC GCT GGA
leu gln gly leu lys ser cys his thr gly leu arg arg thr ala gly
 129
450
TGG AAT GTG CCT ATA GGG ACA CTT CGT CCA TTC TTG AAT TGG ACG GGT
trp asn val pro ile gly thr leu arg pro phe leu asn trp thr gly
 145
```

Sheet 1 of 4

FIGURE 6

498
CCA CCT GAG CCC ATT GAG GCA GCT GTG GCC AGG TTC TTC TCA GCC AGC
pro pro glu pro ile glu ala ala val ala arg phe phe ser ala ser
161
546
TGT GTT CCC GGT GCA GAT AAA GGA CAG TTC CCC AAC CTG TGT CGC CTG
cys val pro gly ala asp lys gly gln phe pro asn leu cys arg leu
177
594
TGT GCG GGG ACA GGG GAA AAC AAA TGT GCC TTC TCC TCC CAG GAA CCG
cys ala gly thr gly glu asn lys cys ala phe ser ser gln glu pro
193
642
TAC TTC AGC TAC TCT GGT GCC TTC AAG TGT CTG AGA GAC GGG GCT GGA
tyr phe ser tyr ser gly ala phe lys cys leu arg asp gly ala gly
209
690
GAC GTG GCT TTT ATC AGA GAG AGC ACA GTG TTT GAG GAC CTG TCA GAC
asp val ala phe ile arg glu ser thr val phe glu asp leu ser asp
225
738
GAG GCT GAA AGG GAC GAG TAT GAG TTA CTC TGC CCA GAC AAC ACT CGG
glu ala glu arg asp glu tyr glu leu leu cys pro asp asn thr arg
241
786
AAG CCA GTG GAC AAG TTC AAA GAC TGC CAT CTG GCC CGG GTC CCT TCT
lys pro val asp lys phe lys asp cys his leu ala arg val pro ser
257
834
CAT GCC GTT GTG GCA CGA AGT GTG AAT GGC AAG GAG GAT GCC ATC TGG
his ala val val ala arg ser val asn gly lys glu asp ala ile trp
273
882
AAT CTT CTC CGC CAG GCA CAG GAA AAG TTT GGA AAG GAC AAG TCA CCG
asn leu leu arg gln ala gln glu lys phe gly lys asp lys ser pro
289
930
AAA TTC CAG CTC TTT GGC TCC CCT AGT GGG CAG AAA GAT CTG CTG TTC
lys phe gln leu phe gly ser pro ser gly gln lys asp leu leu phe
305
978
AAG GAC TCT GCC ATT GGG TTT TCG AGG GTG CCC CCG AGG ATA GAT TCT
lys asp ser ala ile gly phe ser arg val pro pro arg ile asp ser
321
1026
GGG CTG TAC CTT GGC TCC GGC TAC TTC ACT GCC ATC CAG AAC TTG AGG
gly leu tyr leu gly ser gly tyr phe thr ala ile gln asn leu arg
337

```
1074
   AAA AGT GAG GAG GAA GTG GCT GCC CGG CGT GCG CGG GTC GTG TGG TGT
   lys ser glu glu glu val ala ala arg arg ala arg val val trp cys
   353
1122
   GCG GTG GGC GAG CAG GAG CTG CGC AAG TGT AAC CAG TGG AGT GGC TTG
   ala val gly glu gln glu leu arg lys cys asn gln trp ser gly leu
   369
1170
   AGC GAA GGC AGC GTG ACC TGC TCC TCG GCC TCC ACC ACA GAG GAC TGC
   ser glu gly ser val thr cys ser ser ala ser thr thr glu asp cys
   385
1218
   ATC GCC CTG GTG CTG AAA GGA GAA GCT GAT GCC ATG AGT TTG GAT GGA
   ile ala leu val leu lys gly glu ala asp ala met ser leu asp gly
   401
1266
   GGA TAT GTG TAC ACT GCA GGC AAA TGT GGT TTG GTG CCT GTC CTG GCA
   gly tyr val tyr thr ala gly lys cys gly leu val pro val leu ala
   417
1314
   GAG AAC TAC AAA TCC CAA CAA AGC AGT GAC CCT GAT CCT AAC TGT GTG
   glu asn tyr lys ser gln gln ser ser asp pro asp pro asn cys val
   433
1362
   GAT AGA CCT GTG GAA GGA TAT CTT GCT GTG GCG GTG GTT AGG AGA TCA
   asp arg pro val glu gly tyr leu ala val ala val val arg arg ser
   449
1410
   GAC ACT AGC CTT ACC TGG AAC TCT GTG AAA GGC AAG AAG TCC TGC CAC
   asp thr ser leu thr trp asn ser val lys gly lys lys ser cys his
   465
1458
   ACC GCC GTG GAC AGG ACT GCA GGC TGG AAT ATC CCC ATG GGC CTG CTC
   thr ala val asp arg thr ala gly trp asn ile pro met gly leu leu
   481
1506
   TTC AAC CAG ACG GGC TCC TGC AAA TTT GAT GAA TAT TTC AGT CAA AGC
   phe asn gln thr gly ser cys lys phe asp glu tyr phe ser gln ser
   497
1554
   TGT GCC CCT GGG TCT GAC CCG AGA TCT AAT CTC TGT GCT CTG TGT ATT
   cys ala pro gly ser asp pro arg ser asn leu cys ala leu cys ile
   513
1602
   GGC GAC GAG CAG GGT GAG AAT AAG TGC GTG CCC AAC AGC AAT GAG AGA
   gly asp glu gln gly glu asn lys cys val pro asn ser asn glu arg
   529
```

1650
TAC TAC GGC TAC ACT GGG GCT TTC CGG TGC CTG GCT GAG AAT GCT GGA
tyr tyr gly tyr thr gly ala phe arg cys leu ala glu asn ala gly
545
1698
GAC GTT GCA TTT GTG AAA GAT GTC ACT GTC TTG CAG AAC ACT GAT GGA
asp val ala phe val lys asp val thr val leu gln asn thr asp gly
561
1746
AAT AAC AAT GAG GCA TGG GCT AAG GAT TTG AAG CTG GCA GAC TTT GCG
asn asn asn glu ala trp ala lys asp leu lys leu ala asp phe ala
577
1794
CTG CTG TGC CTC GAT GGC AAA CGG AAG CCT GTG ACT GAG GCT AGA AGC
leu leu cys leu asp gly lys arg lys pro val thr glu ala arg ser
593
1842
TGC CAT CTT GCC ATG GCC CCG AAT CAT GCC GTG GTG TCT CGG ATG GAT
cys his leu ala met ala pro asn his ala val val ser arg met asp
609
1890
AAG GTG GAA CGC CTG AAA CAG GTG CTG CTC CAC CAA CAG GCT AAA TTT
lys val glu arg leu lys gln val leu leu his gln gln ala lys phe
625
1938
GGG AGA AAT GGA TCT GAC TGC CCG GAC AAG TTT TGC TTA TTC CAG TCT
gly arg asn gly ser asp cys pro asp lys phe cys leu phe gln ser
641
1986
GAA ACC AAA AAC CTT CTG TTC AAT GAC AAC ACT GAG TGT CTG GCC AGA
glu thr lys asn leu leu phe asn asp asn thr glu cys leu ala arg
657
2034
CTC CAT GGC AAA ACA ACA TAT GAA AAA TAT TTG GGA CCA CAG TAT GTC
leu his gly lys thr thr tyr glu lys tyr leu gly pro gln tyr val
673
2082
GCA GGC ATT ACT AAT CTG AAA AAG TGC TCA ACC TCC CCC CTC CTG GAA
ala gly ile thr asn leu lys lys cys ser thr ser pro leu leu glu
689
2130
GCC TGT GAA TTC CTC AGG AAG TAA
ala cys glu phe leu arg lys *** ACCGAA GAAGATGGCC CAGCTCCCCA
705
2180
AGAAAGCCTC AGCCATTCAC TGCCCCCAGC TCTTCTCCCC AGGTGTGTTG GGGCCTTGGC

2240
TCCCCTGCTG AAGGTGGGGA TTGCCCATCC ATCTGCTTAC AATTCCCTGC TGTCGTCTTA

2300
GCAAGAAGTA AAATGAGAAA TTTTGTTGAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/03614

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 15/06, 15/14; C12N 15/04, 15/06, 15/10, 15/12
US CL :536/23.5; 530/350, 394; 435/69.1, 240.2, 254, 255, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 530/350, 394; 435/69.1, 240.2, 254, 255, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, Swissprot, PIR, search terms: lactoferrin, amylase, oryzae, aspergillus, cDNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Biochemical Journal, Volume 276, issued June 1991, Stowell et al., "Expression of cloned human lactoferrin in baby-hamster kidney cells", pages 349-355, see especially p. 350, column 2.	1,3,4,8,10,11,13, 16-18,21 1-21
Y	WO, A, 89/01969 (Woldike, H.F.) 09 March 1989, see whole publication, especially pages 3, 5, 6, 10 and 11.	1-21
Y	Gene, Volume 79, issued 1989, Gines et al., "Aspergillus oryzae has two nearly identical Taka-amylase genes, each containing eight introns", pages 107-117, see especially the abstract and Figures 2 and 3.	1-21
Y	Journal of Molecular Biology, Volume 173, issued 1984, G. von Heijne, "How signal sequences maintain cleavage specificity", pages 243-251, see especially page 244.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1993	27 JUL 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer KEITH C. FURMAN, PH.D.
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 18, Number 13, issued 1990, Powell et al., "Nucleotide sequence of human lactoferrin cDNA", page 4013.	1-21
Y	Lipids, Volume 24, Number 9, issued 1989, Huge-Jensen et al., "Rhizomucor miehei triglyceride lipase is processed and secreted from transformed Aspergillus oryzae", pages 781-785, see especially the abstract.	1-21
Y	US, A, 4,740,461 (Kaufman) 26 April 1988, see whole patent, especially Table 1 and columns 11 and 12.	1-21
Y,P	US, A, 5,155,037 (Summers) 13 October 1992, filed 04 August 1989, see whole patent, especially columns 1 and 4-8.	1, 3, 8, 10, 11, 12, 16-18, 21